Radiotherapy of human sarcoma promotes an intratumoral immune effector signature

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Abstract: PURPOSE: The tumor immune microenvironment plays a crucial role in the development and progression of cancer. Sarcomas are a group of heterogeneous soft tissue malignancies that are often treated with radiotherapy as a part of the treatment concept. There is increasing evidence that radiotherapy leads to alterations in the tumor microenvironment, particularly with respect to the immune infiltrate. The present study has been carried out to develop a better understanding of such changes following radiotherapy. EXPERIMENTAL DESIGN: We retrospectively analyzed the expression of 35 immune response-related genes by qRT-PCR analysis and immunohistochemistry on paired formalin-fixed paraffin-embedded tumor samples from 38 sarcoma patients before and after radiotherapy. RESULTS: We observed that radiotherapy results in a significant upregulation of several immune effectors and cancer-testis antigens and a concomitant downregulation of immune suppressors, indicating that radiotherapy may support the immune defense in sarcomas. CONCLUSIONS: These novel findings may have implications for the design of therapeutic regimens which exploite the immune system in sarcoma patients by combining standard radiotherapy with immunotherapeutic strategies.

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Radiotherapy of human sarcoma promotes an intratumoral immune effector signature

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Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest

ABSTRACT

Purpose: The tumor immune microenvironment plays a crucial role in the development and progression of cancer. Sarcomas are a group of heterogeneous soft tissue malignancies that are often treated with radiotherapy as a part of the treatment concept. There is increasing evidence that radiotherapy leads to alterations in the tumor microenvironment, particularly with respect to the immune infiltrate. The present study has been carried out to develop a better understanding of such changes following radiotherapy.

Experimental Design: We retrospectively analyzed the expression of 35 immune response-related genes by qRT-PCR analysis and immunohistochemistry on paired formalin-fixed paraffin-embedded tumor samples from 38 sarcoma patients before and after radiotherapy.

Results: We observed that radiotherapy results in a significant upregulation of several immune effectors and cancer-testis antigens and a concomitant downregulation of immune suppressors, indicating that radiotherapy may support the immune defense in sarcomas.

Conclusions: These novel findings may have implications for the design of therapeutic regimens which exploite the immune system in sarcoma patients by combining standard radiotherapy with immunotherapeutic strategies.

Translational Relevance

Sarcomas are cancers with low incidence but high mortality, which are often treated with radiotherapy. However, very little is known about the impact of radiotherapy on the quality of the tumor-associated immune infiltrate in human sarcomas. The current study set out to investigate such changes using paired tumor samples before and after radiotherapy from a cohort of 38 sarcoma patients. Our findings demonstrate that radiotherapy may groom the local tumor microenvironment by promoting an

intratumoral immune effector signature in patients who survived for three years or more following radiotherapy. Furthermore, radiotherapy resulted in upregulated expression of MHC class I molecules and cancer-testis antigen. This knowledge argues for combining radiotherapy with immunotherapy as a novel modality for the treatment of sarcoma.

INTRODUCTION

Sarcomas are a heterogeneous group of malignant tumors of mesenchymal origin. They can arise anywhere in the bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue (1). Sarcomas comprise 1% of all adult cancers and about 15% of all childhood cancers there, with high mortality and metastatic potential (2). There is clear evidence from patients and preclinical models that the immune system can control the development of malignancies, a concept termed tumor immunoediting and immunosurveillance (3). Moreover, spontaneous tumor-specific immunity can be detected in cancer patients and in tumor-bearing mice (4, 5). The targets of such responses are usually tumor-associated antigens, of which the group of cancer-testis (CT) antigens is a prominent representative (6). CT-antigens are especially interesting because they are highly immunogenic and as their name suggests, their expression is restricted to germ cells and cancer cells. More than 100 CT genes are known to date (7, 8). However, when tumor-specific immunity fails to eliminate cancer, it has proven very difficult to boost this response such that tumor control is achieved. The adaptive arm of the immune response consists of T and B lymphocytes, including antibodies, $CD8^+$ T cells that recognize tumor antigens presented by MHC class I (MHC-I) molecules on tumor cells (9) are thought to be major effectors. Natural killer (NK) cells, which represent the innate immune system, may also mediate antitumor effects by direct cytotoxicity towards malignant cells that

express low levels of MHC-I and by producing immunostimulatory cytokines such as IFN-γ (10). Immune effector functions, however, are hampered by an immunosuppressive environment, which is created by the tumor and to which $FoxP3^+$ regulatory T cells as well as myeloid cells contribute through a variety of mechanisms including TGFβ, arginase-1 and IL-10 (11-13). The tumor microenvironment presumably significantly influences the outcome of conventional cancer therapies (14, 15). Human sarcomas are treated by radiotherapy, chemotherapy and/or surgery (16). Recent evidence suggests that both radio- and chemotherapy result in the mobilization of innate and adaptive immunity (17-19), but very little is known about the impact of radiotherapy on the immune infiltrate in human sarcomas. We investigated the effect of radiotherapy on the tumor microenvironment in sarcoma patients using paired biopsies that were collected before and after radiotherapy from 38 sarcoma patients. Upon radiotherapy we found an increase of many cell types and molecules that are characteristic for protective immunity, whereas those associated with immunoregulation were often downregulated. Furthermore, we found a relatively high expression of immune effectors and low expression of immune suppressors after radiotherapy in those patients who survived for three years or more after radiotherapy in comparison to those who survived for less than three years and died. Our results justify further investigating whether a combination of radiotherapy and immune stimulation results in a better clinical outcome than either therapy alone.

MATERIALS AND METHODS

Patient material

Formalin-fixed, paraffin-embedded (FFPE) paired tumor samples were collected from a cohort of 38 sarcoma patients before and after radiotherapy. Tumor samples were retrieved from the archives of the Department of Pathology, University Hospital Zurich. The samples after radiotherapy were obtained from planned resection and

the time points of collection following radiotherapy varied between 1-89 days (Supplementary Table S1). Patients with non-metastatic disease and only those patients who received neo-adjuvant radiotherapy before surgery as a part of their standard treatment regimen were included in the study. The patients were treated by standard photon-radiotherapy and received a cumulative dose of 40-60 Gy (2 Gy per treatment). An overview of the sarcoma subtypes, cumulative radiation dose, percentage of dead cells following radiotherapy and clinical outcome is given in Table 1. The ethics committee "Ethical Committee of the Canton of Zurich" specifically approved this study (EK-2011-0224/0). For the present study, all relevant data were retrieved from the computerized database of the Department of Pathology, University Hospital Zurich and transferred into a separate anonymous database. Fifteen patient samples from our previous work overlapped in the current study (17), but were specifically approved under the study (EK-2011-0224/0) All patients signed the informed consent form in accordance with the declaration of Helsinki and were given a unique code, thus protecting the patient identity.

RNA isolation and reverse transcription

Three punches (0.6 mm diameter) were randomly taken from each of the FFPE blocks using a tissue arrayer MTA-1 (Beecher Instruments). Deparaffinization was performed in 300 µL elution buffer (1 M Tris pH 8, 0.5 M EDTA pH 8, 20% SDS (all Ambion), ultrapure water (Sigma)) at 95 °C for 10 min on a shaker followed by centrifugation at 4°C, 14'000 rpm for 10 min, followed by digestion with 3 µL of Proteinase K (18 +/- 4 mg/mL, Roche) at 55°C for 72 h. The samples were then centrifuged at 4°C, 14'000 rpm for 2 min. The supernatants (250 µL) were transferred into fresh tubes and 750 µL TriZol LS reagent (Invitrogen) was added to each sample while vortexing followed by homogenization by centrifugation at 4°C, 14'000 rpm for 2 min using QIA-shredder columns. This was followed by RNA purification using phenol and chloroform. Two hundred µL of chloroform was added to each flow-

through with mild vortexing, followed by incubation at room temperature for 5 min until the two phases separated, followed by centrifugation at 4°C, 14'000 rpm for 15 min. The upper aqueous phase containing the RNA was transferred into a new tube, 20 µg glycogen (Invitrogen) was added followed by the precipitation of RNA by adding 0.5 mL isopropanol (99.9% v/v, Kantonsapotheke Zürich). Samples were incubated at room temperature for 15 min and then centrifuged at 4°C, 14'000 rpm for 20 min. The supernatant was removed and the pellet was washed with 1 mL 75% ethanol (Merck), air-dried, dissolved in RNase free water (Sigma) and digested with 80 U/mL DNAse I (New England Biolabs) at room temperature for 15 min, followed by inactivation using 2 mM EDTA (Ambion) at 65°C for 10 min. The concentration and purity was evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Three hundred ng of RNA was reverse transcribed using the high-capacity cDNA Reverse Transcription Kit (Applied Biosciences). The cDNA was either used immediately for qRT-PCR reactions or stored at -20°C until use. Because of limited material availability the cDNA was preamplified. Preamplification was performed for 14 cycles using TaqMan® PreAmp Master Mix Kit (Applied Biosystems). All kits were used according to the manufacturer's instructions.

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

The expression of target genes was analyzed using TaqMan® gene expression assays containing commercially available predeveloped TaqMan reagents with optimized primer and probe concentrations and TaqMan 1x universal master mix (Applied Biosystems) (Supplementary Table S2), on a RotoGene Q real-time PCR cycler (Qiagen). The reaction mixture (10 µL) consisted of 1 µL cDNA, 3.5 µL water, 0.5 µL primer and 5 µL TaqMan 1x universal master mix. The following cycle conditions were used: initial hold for 2 min at 50°C and 10 min at 95°C the probes were cycled 45 times at 95°C for 15 sec and at 60°C for 1 min. All reactions were

performed in at least triplicates. Threshold cycle (Ct) values were determined using the Rotor-Gene Q Series software 1.7. ΔCt levels of the transcripts were calculated by normalization to the endogenous control (β-actin). The change in expression (ΔΔCt) levels of the target genes following radiotherapy is given as the fold change in expression ($2^{-(\Delta\Delta Ct)}$) and is determined as [$\Delta Ct_{\text{before radiotherapy}} - \Delta Ct_{\text{after radiotherapy}}$]. Ct values >38 cycles were interpreted as negative for gene expression. Fold changes in the range of a fold increase of >0.5 and <2 was considered as not significant. This range is marked as shaded area in the figures.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on FFPE paired tissue sections obtained from a cohort of 37-38 sarcoma patients. Sections were stained with mouse anti-human monoclonal antibodies against CD4 (clone 1F6, 1:30, ZYMED Laboratories Inc.), CD8 (clone C8/114B, 1:100, DAKO A/S), MHC-I (clone C21, 1:1000, RDI Research Diagnostics, Inc.), CD3 (clone F7.2.38, 1:50, Dako), CT7 (clone CT7-33, 1:80, DAKO A/S), NY-ESO-1 (clone E978, 1:50, ZYMED). Sections were counterstained with hematoxylin, dehydrated and mounted. All sections were processed with the Ventana Benchmark automated staining system (Ventana Medical Systems) using Ventana reagents for NY-ESO-1, CT7, CD4, CD8 and CD3 and the BondMax (Vison BioSystems) for MHC-I. UView (Ventana) or Refine DAB (Vision BioSystems) were used as chromogens against the primary antibodies. The slides were scanned on a NDP.view2 software (Hamamatsu), and analyzed using the IHC analysis software (NDP.analyze, Hamamatsu). The fields were observed at a magnification of × 200 (field of view = 493.1 × 368.5 μm^{-1}). The IHC analysis was performed by counting 10 random fields. The staining was scored on a scale of 0 to 5 for the expression of NY-ESO-1 and MAGE-C1/CT7 as a percentage, based on the number of positive cells expressing the antigen to the total number of cells in a high-

power field (HPF). The tumor infiltrates were calculated as the number of cells expressing CD3, CD4, and CD8 per HPF. The MHC-I sections were acquired on Zeiss-Axiovert 200M (Carl Zeiss Light Microscopy) inverse microscope using Carl Zeiss Axiovision CD28 imaging system. The scoring for MHC-I expression was done based on the intensity of staining (details in Supplementary Table S3). Two individuals performed the scoring blindly and independently for MHC-I and one individual performed the scoring blindly for the other stainings. The IHC staining was interpreted in conjunction with a single haematoxylin and eosin (H&E) stained slide by a pathologist (BB). The malignant nature of the tumors for the IHC evaluation and qRT-PCR analysis was apparent from the H&E stained slides.

Statistical analysis and heatmaps

Correlations between the different immune response related transcripts was assessed using the Spearman`s rho correlation coefficient (ρ) and the significance-two tailed analysis (P value) was used to analyse the statistical significance (IBM SPSS statistics software). The dot plots for all significant correlations were generated using the Graphpad-Prism software. Statistical significance was defined as $P < 0.05$.

For visualizing the gene expression profile in the sarcoma patients following radiotherapy, heatmaps were generated using Software: R, Bioconductor library "gplots", heatmap.2 software. Subtracting the mean expression value across patients centered the values for each transcript. No gene-specific scaling (standardization) was done, so that the information about the relative signal strength between probes remained intact. The color tone in the heatmaps was calibrated, so that saturated red (upregulation) and saturated green (downregulation) were reached at values equal to three times the standard deviation of the expression values of the entire matrix. Heatmaps were created for all the patients using the $\Delta\Delta$Ct values [$\Delta Ct_{\text{before radiotherapy}} - \Delta Ct_{\text{after radiotherapy}}$]. For the purpose of clustering the genes, the *de novo* expression

was assigned a ΔΔCt value of -10 and the loss of expression was assigned a ΔΔCt value of +10. A hierarchical clustering method using Software: R, Bioconductor library "gplots", heatmap.2 software was used to group genes on the basis of similar expression patterns, in relation to their survival status (green indicates similar behavior of genes and red indicates the reverse). Statistical analysis was performed for each transcript between the cohort before and after radiotherapy using the two-tailed bivariate analysis (P value) (Graphpad-Prism) on the ΔCt values after normalization to β-actin.

RESULTS

Radiotherapy results in upregulation of transcripts of several immune effector molecules and CT-antigens and downregulation of transcripts associated with immune suppression in sarcoma patients

To understand the immunomodulatory effects of radiotherapy in sarcomas, we retrospectively analyzed the expression of several immune response-related transcripts and CT-antigens (Supplementary Table S2) by qRT-PCR analysis on paired formalin-fixed paraffin-embedded (FFPE) tumor tissues from 37 sarcoma patients before and after radiotherapy (Table 1). We observed that radiotherapy resulted in upregulated transcripts specific for several immune effector cells or proteins including CD45, CD3, CD4, CD8, MHC-I, MHC-II, perforin, granzyme B, IFN-γ, IL-12 and NKG2D in most patients. Additionally, we observed *de novo* expression of perforin, NKG2D and iNOS in two or more patients. TNF-α transcripts were upregulated in several patients but downregulated in others. Concomitantly, the expression of various transcripts related to immune suppression (FoxP3, CD68, CD163, CTLA-4, arginase-1, IDO, PD-1, PD-L1, IL-10, TGF-β and BTLA) was downregulated in some patients but upregulated or unchanged in others. At least in

one of the patients the expression of FoxP3, PD-1, CTLA-4, BTLA or IL-10 was lost following radiotherapy (Fig. 1A and B, Table 2). Additionally, radiotherapy resulted in a substantial upregulation or *de novo* expression of one or more CT-antigens (CT7, LAGE-A1, MAGE-A9 and NY-ESO-1) (Fig. 1C, Table 2). As the fold change in expression in terms of *de novo* or loss of gene expression cannot be computed, the grand mean was calculated excluding these values. Our data suggest that radiotherapy results in a favourable upregulation of transcripts associated with effector function when compared to those associated with immune suppression, therefore indicating that radiotherapy may presumably promotes protective immunity in the tumor (Table 2 and Supplementary Fig. S4).

Radiotherapy results in increased expression of CT-antigens, MHC-I and in lymphocyte infiltration at the protein level

To validate our findings at the protein level we compared the expression of CT-antigens, MHC-I as well as the infiltration of lymphocytes by immunohistochemistry in 37-38 paired FFPE sections obtained from sarcoma patients before and after radiotherapy. Radiotherapy upregulated the expression of MHC-I in 30 of 38 patients (Fig. 2D) and resulted in an increased infiltration by $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 2A-C, Table 3). Furthermore, radiotherapy resulted in a substantial upregulation or *de novo* expression of the CT-antigens NY-ESO-1 in 13 of 38 patients (Fig. 2E, Table 3) and of CT7 in 17 of 38 patients (Fig. 2F, Table 3). Interestingly, most of the patients with an upregulated expression of one or more CT-antigens had a concomitant upregulation of MHC-I and/or increased infiltration of T cells (patient number: 2, 3, 5, 7, 8, 9,10, 11, 15, 17,19, 20, 22, 23 25, 27, 31, 33, 34, 35, and 36), which approximately comprises 55.3% of the patients. Those changes following radiotherapy were significant when compared to the untreated samples (Supplementary Fig. S5). Due to limited availability of material, we could not perform staining for additional CT-antigens. We know from our previous work, that the

expression of CT-antigens is upregulated upon irradiation in different tumor types (17). Representative sections (Fig. 2G) illustrate that there is a difference in the degree of radiotherapy-induced changes between individual sarcoma patients. A particular gene may be homogeneously expressed after radiotherapy (lower panels), whereas its expression may be upregulated, but still heterogeneous in others (upper panels). The concomitant upregulated expression of MHC-I and CT-antigens upon radiotherapy may increase the visibility of the tumor for $CD8^+$ T cells.

Radiotherapy results in clustered changes in expression of genes associated with immune suppression or immune effector function.

To investigate whether the changes we observed upon radiotherapy are part of a coordinated program we plotted our data as a heatmap by computing the ΔΔCt values of each of the transcripts against the ΔΔCt value of the other transcripts. Transcripts associated with immune suppression including IDO, BTLA, FoxP3, PD-L1, IL-10, TGF-β, STAT-3, CT10, TNF-α and iNOS seem to follow a similar pattern of downregulated expression after radiotherapy. Furthermore, transcripts associated with immune effector function such as NKG2D, CD45, CD3, CD4, CD8, MHC-II, β2M, perforin, granzyme B, the CT-antigen CT7 and macrophages (CD68, CD163) seem to be upregulated in a coordinated fashion following radiotherapy (Fig. 3A). In order to determine the significance of correlation between two target genes, the ΔΔCt values of each of the target genes were computed against the other using a two-tailed bivariate analysis and the Spearman`s rho correlation coefficient. For mathematical reasons, we excluded patients with *de novo* expression or loss of expression following radiotherapy from the analysis. We found a positive correlation between several immune effectors and an inverse correlation between the transcripts of several immune effectors and immune suppressors. The scattered dot plots for the significant correlations are represented in Supplementary Figure S6. The

Spearman`s rho correlation coefficient for all the correlations is listed in the Supplementary Table S7.

Radiotherapy results in downregulation of genes associated with immune suppression in sarcoma patients with prolonged survival

To correlate the gene expression profile in sarcoma patients following radiotherapy in relation to their survival status, we plotted the change in expression ($\Delta\Delta$Ct) levels of the target genes following radiotherapy in a heatmap. We excluded the CT-antigen SSX-2 from this analysis, because it was expressed in only two tumor samples. All patients who survived for three or more years after radiotherapy, irrespective of the clinical outcome at the end of the follow up, formed one group. Those who survived for less than three years after radiotherapy and were dead at the end of the follow up formed the other group. The heatmap reveals a clear downregulation of genes associated with immune suppression such as arginase-1, BTLA, CTLA-4, IDO, iNOS, PD-1, PD-L1, IL-10, TGF-β, TNF-α, FoxP3, macrophages (CD68, CD163) and CT10 in samples of patients who survived for a period of three years or more after radiotherapy, but not in those who died. The immune effector genes followed a more homogenous pattern of expression independent of the survival status following radiotherapy (Fig. 4).

DISCUSSION

Radiotherapy is an integrated treatment option for sarcoma patients. For considerable time it was thought that radiotherapy may have immunosuppressive effects (20), however evidence from recent animal studies suggests that radiotherapy supports local anti-tumor immunity (18, 21, 22). The current study demonstrates in human sarcoma patients that radiotherapy indeed changes the local tumor

microenvironment from immunosuppressive into one that is reminiscent of protective immunity. We compared the immune infiltrate in paired biopsies from 38 human sarcoma patients collected before and after radiotherapy and found that radiotherapy induced remarkable changes, all of which suggest a skewing towards protective effector immunity. The activation of $CD8^+$ T and $CD4^+$ T cells contributes to anti-tumor immunity (23, 24) and is also known to facilitate the efficacy of radiotherapy (25, 26); this is clearly supported by a higher tumor incidence in immunosuppressed patients (27). We observed a higher infiltration of both $CD4^+$ and $CD8^+$ T cells in most of the sarcoma patients after radiotherapy. Additionally, we observed that radiotherapy resulted in an upregulated expression of MHC-I and of different CT-antigens in most sarcoma patients, which makes tumors more visible to $CD8^+$ T cells. These data are in accordance with published data showing that non-lethal doses of radiation induce a dose-dependent increase of MHC-I (28) and CT-antigens resulting in better recognition by specific $CD8^+$ T cells *in vitro* (17). Importantly, radiotherapy sometimes changed the heterogeneous expression of MHC-I and/or CT-antigens, which is thought to result from immune escape (3, 29), to a homogeneous expression, thus enabling a more complete surveillance of the tumor. In addition to changes that improve immunological visibility of tumor cells, we also detected *de novo* expression of the highly immunogenic CT-antigens in some patients as a result of radiotherapy; this is well in line with our previous data (17). The observed changes are relevant as the loss of MHC-I expression in the equilibrium phase is thought to contribute to escape from immune control leading to tumor progression (30).

The tumor microenvironment is often immunosuppressive, thereby failing to activate local immune responses (31). Several studies have described the roles of immunosuppressive cytokines such as TGF-β and IL-10 in aiding tumor progression and that of stimulatory cytokines such as TNF-α, IL-12 and IFN-γ in supporting anti-tumor effects (32-36). We observed a higher expression of immune effector

molecules, including perforin, granzyme B, NKG2D, IFN-γ, TNF-α and IL-12, some of which were only expressed after radiotherapy in particular patients. At the same time many molecules and cell types associated with immunosuppression, including arginase-1, BTLA, CTLA-4, PD-1, PD-L1, IDO, FoxP3, macrophages, TGF-β and IL-10 were expressed at lower level or even disappeared completely after radiotherapy. In search for immune parameters that predict the response to radiotherapy, we found that radiotherapy-induced downregulation of several immune suppressors correlated with survival status. In addition, the correlation analysis revealed that the transcripts associated with immune suppression (IDO, BTLA, FoxP3, PD-L1, IL-10, TGF-β and macrophages) have a similar pattern of expression. Also immune effectors such as NKG2D, CD45, CD3, CD4, CD8, MHC-II, β2M, perforin and granzyme B respond in a similar and presumably coordinated fashion upon radiotherapy. Despite the clinical heterogeneity in the cohort of sarcoma patients in our study, the response to radiotherapy was similar as were the changes in the immune infiltrate.

The apparent immunostimulation of radiotherapy *per se* argues for combining this therapy with treatments that interfere with immunoregulation and/or enhance immune stimulation. There is evidence in humans and mice that local radiotherapy under CTLA-4 blockade results in tumor regression of non-irradiated tumors as well (abscopal effect) (37, 38). Similar results may be obtained when radiotherapy is combined with blockade of other co-inhibitory molecules, such as Tim-3, PD-1 or BTLA (39-41). A recent study has shown that anti–PD-1 antibody produced objective responses, one in four to one in five patients with advanced nonsmall cell lung cancer, melanoma, or renal-cell cancer (42). This obviously is a promising combination especially in the case of micro-metastases. Another option may be combination radiotherapy with the targeted delivery of immunostimulatory cytokines, which has been shown to synergize with chemotherapy in human and murine cancers (43, 44). A previous study reported, that a combination of radiotherapy with

IL-12 improves both local and distant tumor control compared to either therapy alone (45). Additionally, radiotherapy may create a favorable environment for adoptively transferred tumor-specific T cells (18, 46), which argues for combining adoptive T cell therapy and radiotherapy. Preclinical studies in carcinoembryonic antigen (CEA) transgenic mice and a murine carcinoma cell line transfected with CEA, on combined treatment with low-dose radiation and vaccine expressing CEA and co-stimulatory molecules, could show a 50% reduction in tumor growth and a massive trafficking of T cells, when compared to either treatment alone (47). A clinical trial in cancer patients, vaccinated with NY-ESO-1 DNA vaccine showed induction of antigen-specific T-cell responses in 93% of vaccinated patients, however this did not lead to a clinical benefit (48). A recent study also showed that NY-ESO-1 OLP (overlapping long peptides) vaccine could rapidly induce integrated immune responses (antibody, $CD8^+$ and $CD4^+$) in nearly all vaccinated patients with advanced ovarian cancer, when administered with appropriate adjuvants (49). A Phase I clinical trial in HLA-A2+ melanoma patients using immunization with autologous PBMCs pulsed with a MAGE-3 or a Melan-A peptide, increased specific $CD8^+$ T-cell responses, and six out of eight patients had evidence of clinical activity (50). These studies show that on vaccination with a relevant CT-antigen the peripheral responses could be boosted in cancer patients, however they clearly do not show the desired anti-tumor efficacy. Therefore combining radiotherapy with immune stimulation may support the immune modulating potential of radiotherapy by interfering with the local immunosuppressive milieu. Future trials with a large cohort of patients are required to evaluate the question of clinical outcome and the safety of this combination. This may lead to new concepts in the clinical management of cancer.

Figure Legends

Figure 1. Radiotherapy-induced changes of immune response-related gene expression in human sarcoma. Thirty-seven paired FFPE sarcoma samples before/after radiotherapy were subjected to qR-PCR analysis for 35 different immune response-related genes. (A) Immune cells (B) Cytokines and (C) Cancer Testis antigens. ΔCt levels of the transcripts were calculated by normalization to the endogenous control (β-actin). Results are presented as fold change in expression ($2^{-(\Delta\Delta Ct)}$), which was determined as $[\Delta Ct_{\text{before radiotherapy}} - \Delta Ct_{\text{after radiotherapy}}]$. Genes displayed on the left side of the vertical dotted line are associated with immune effectors and those on the right with immune suppressors. Fold differences in expression within the shaded area are considered as not significant. The bold horizontal line for each transcript represents the grand mean. Each symbol represents an individual patient.

Figure 2. Radiotherapy of human sarcoma results in increased expression of Cancer Testis antigens and MHC-I and promotes infiltration by lymphocytes. Paired FFPE tissue sections from sarcoma patients (n = 37-38) before and after radiotherapy were analyzed by IHC for (A) expression of MHC-I, infiltration of (B) $CD3^+$ (C) $CD4^+$ (D) $CD8^+$ cells, expression of (E) NY-ESO-1 and (F) CT7. Scores of individual patients before (open symbols) and after (closed symbols) radiotherapy are connected with a line. Details to scoring are described in Materials and Methods and are listed in Supplementary Table S3. (G) Representative staining of paired samples before (NR) and after (RT) radiotherapy for MHC-I, CD3, CD4, CD8, CT7 and NY-ESO-1.

Figure 3. Correlation between different immune response-related transcripts following radiotherapy in sarcoma patients (A) Heatmap demonstrating the correlation pattern of immune response-related transcripts in a cohort of paired 37

sarcoma patients following radiotherapy. The histogram indicates gene expression patterns from similar (green) to opposite (red). The white line demarks genes with a similar pattern of expression

Figure 4. Correlation between radiotherapy-induced immunological changes in sarcoma patients and their survival status. Heatmap demonstrating the expression pattern of immune response-related transcripts in sarcoma patients following radiotherapy. The histogram indicates expression level ($\Delta\Delta$Ct values) from low (green) to high (red). The patients are divided into two groups (alive or dead), depending on the three-year survival status. Patients who were alive at the end of the follow up but did not reach the three-year survival mark yet were excluded. The yellow box demarcates a clear difference in gene expression between the alive and dead patients.

Table 1. Patient information. The table summarizes patient diagnosis, cumulative dose of radiation, percentage of cells alive following radiotherapy and the survival status. All patients received only radiotherapy (RT) and underwent surgery as part of their standard treatment regimen. N: number of patients, MPNST: malignant peripheral nerve sheath tumor, NOS: not otherwise specified.

Table 2. Summary of radiotherapy-induced changes in immune response-related transcripts in human sarcomas. A retrospective qRT-PCR analysis of transcripts for (A) immune cells (B) cytokines (C) transcription factors and (D) CT-antigens. ND: not determined, N: number of patients.

Table 3. Summary of radiotherapy-induced changes in infiltration by T cells and expression of MHC-I, CT7 and NY-ESO-1 in human sarcomas. IHC analysis of a cohort of paired tumor samples before/after radiotherapy of 37-38 sarcoma

patients for T cells, MHC-I, CT7 and NY-ESO-1. ND: not determined, N: number of patients.

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Performed the experiments: Sharma. A

Analysis and interpretation of data: Sharma. A, van den Broek. M, Okoniewski. M

Contributed reagents/materials/analytical tools: Bode. B, Moch. H, van den Broek. M, Knuth. A, Okoniewski. M, Sharma. A

Patient information: Bode. B, von Boehmer. L

Writing and review of the manuscript: Sharma. A, van den Broek. M

Histopathological diagnosis: Bode. B, Moch. H

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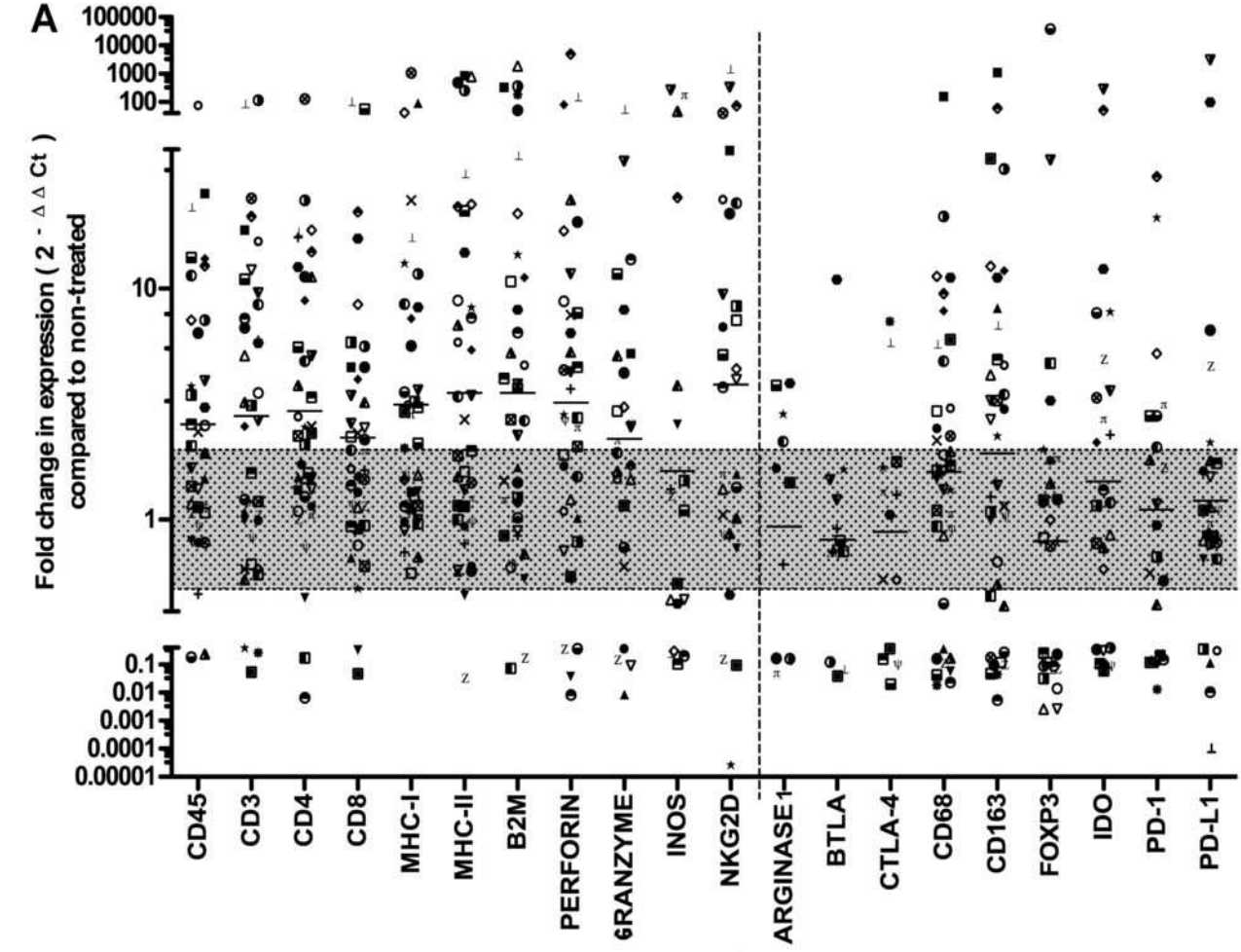
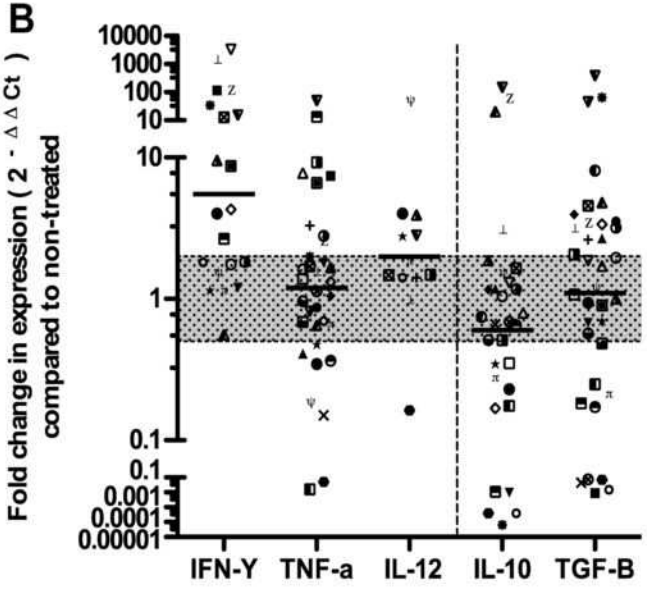
Figure 1.





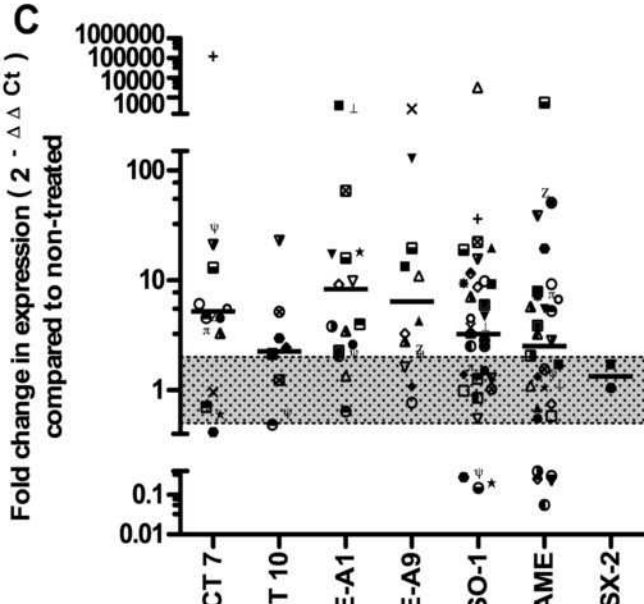
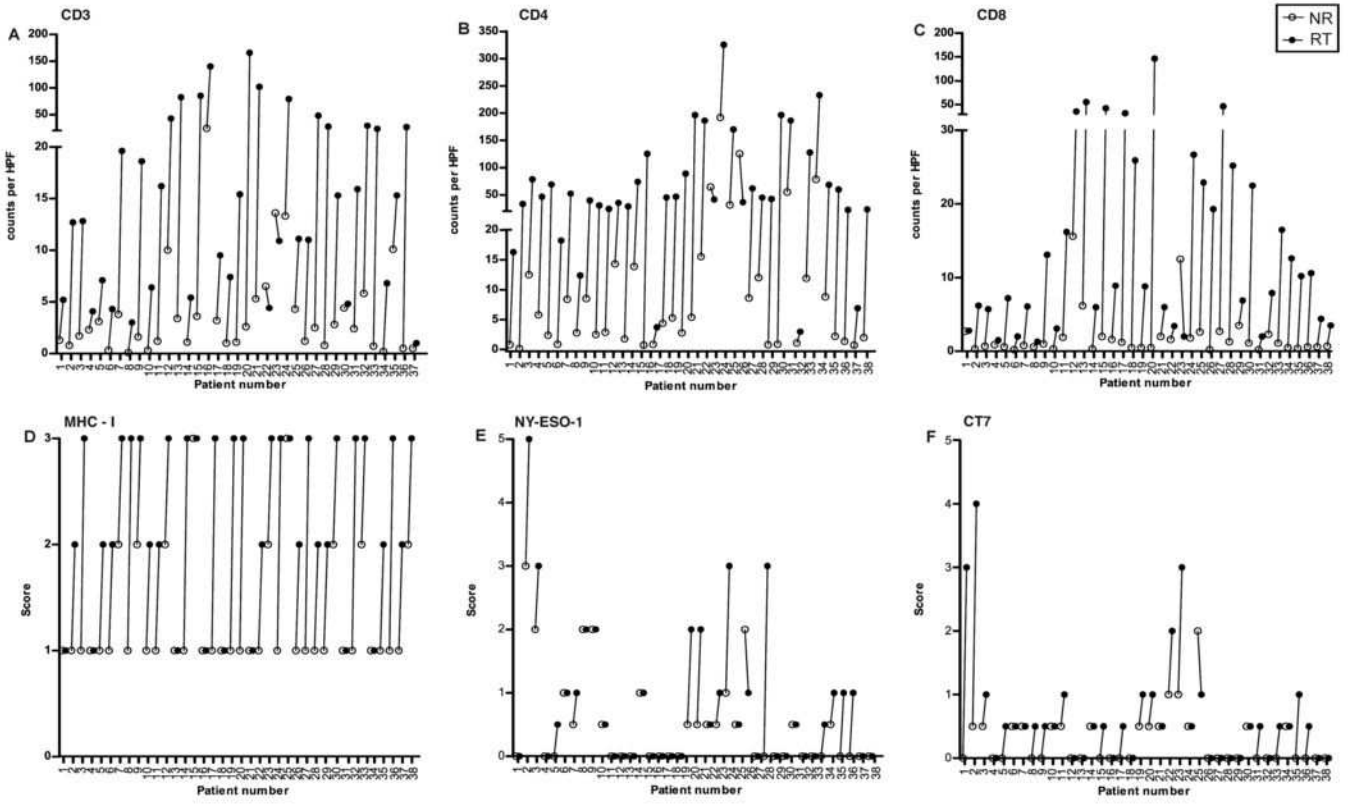


Figure 2



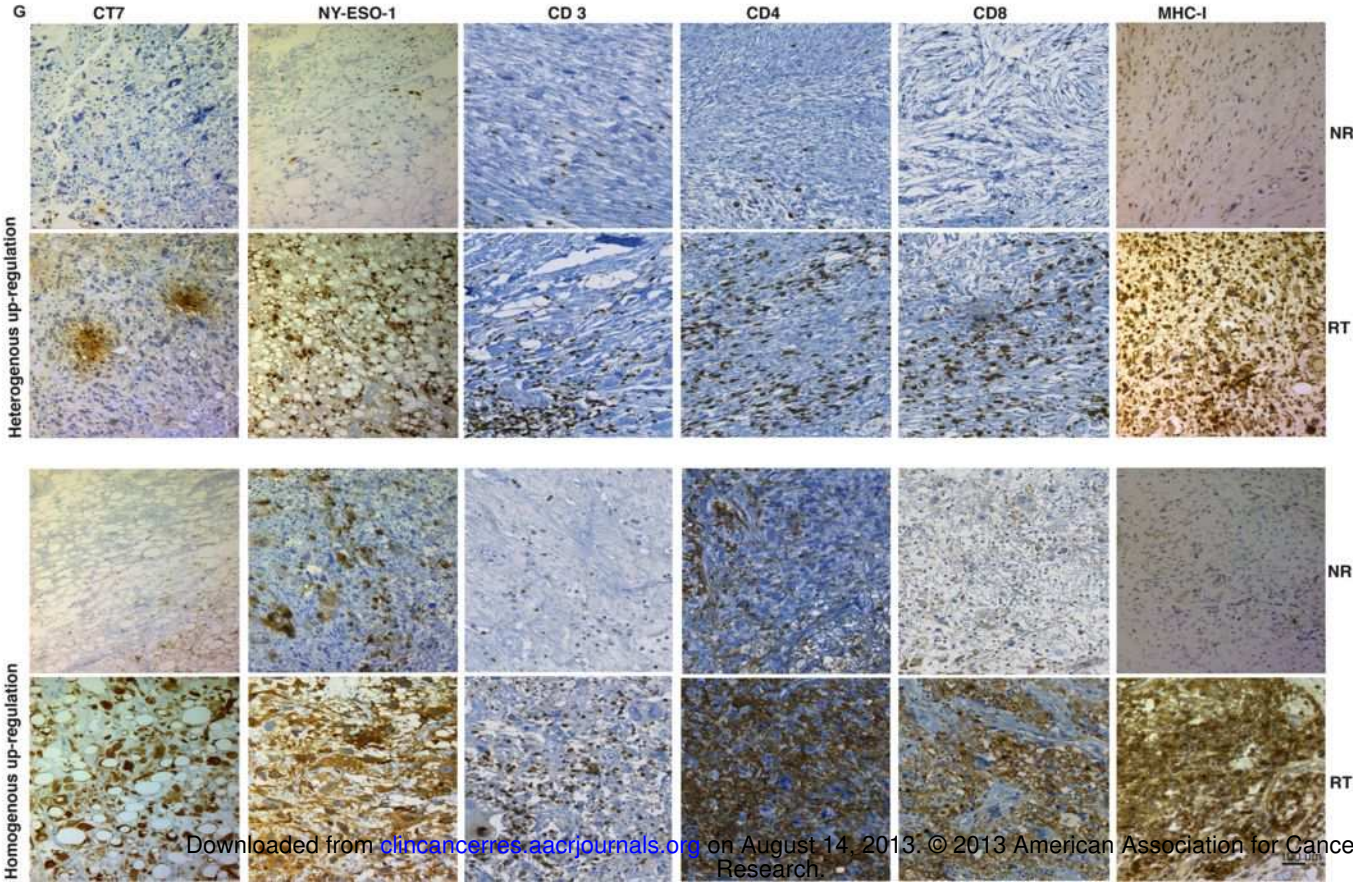


Figure 3

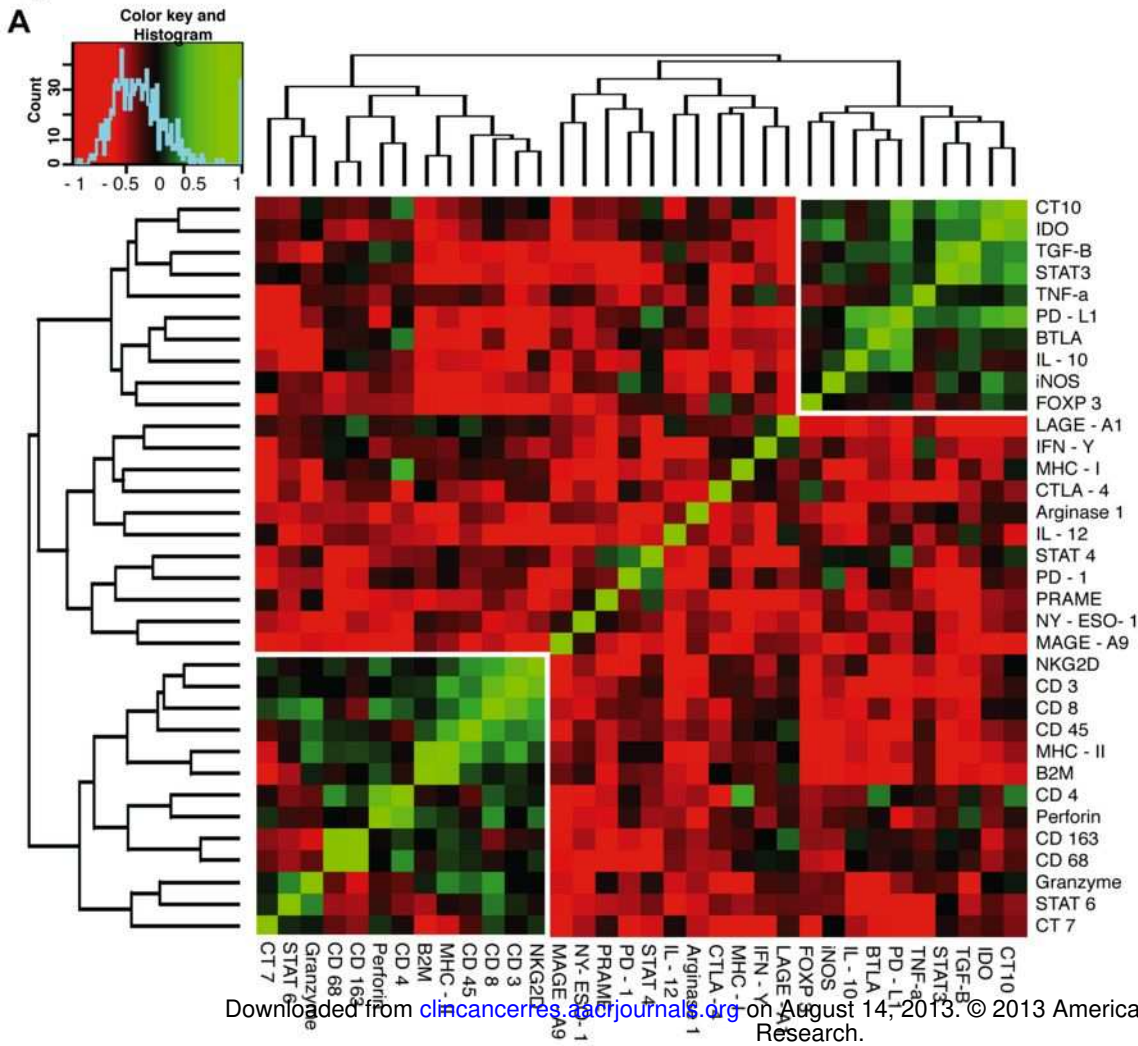


Figure 4

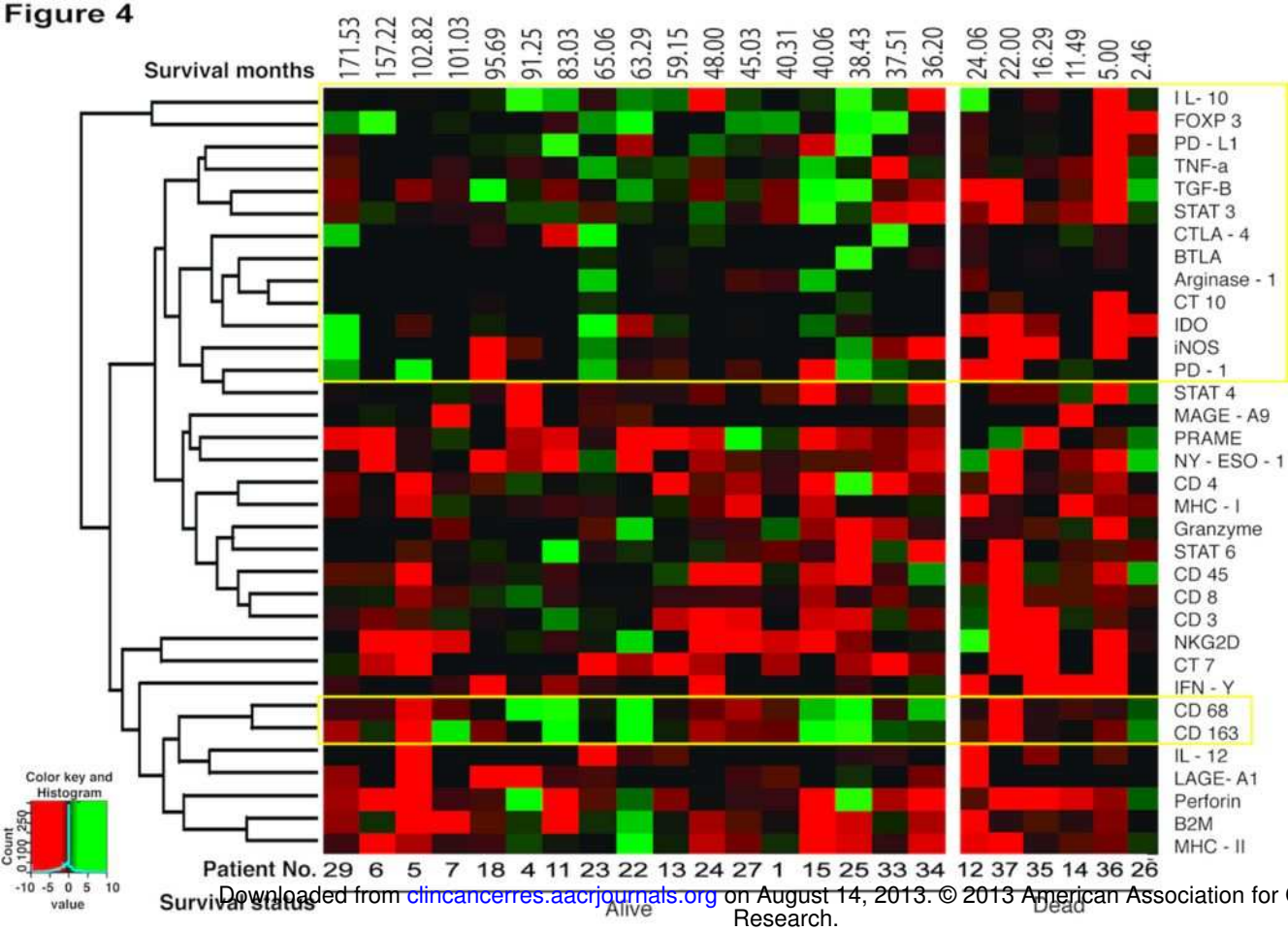


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Table 1

Diagnosis	**N**
Angiosarcoma	1
Leiomyosarcoma	4
MPNST	2
Myxofibrosarcoma	3
Myxoid liposarcoma	10
Pleomorphic liposarcoma	2
Pleomorphic sarcoma NOS	10
Pleomorphic sarcoma with Rhabdomyosarcoma	1
Rhabdomyosarcoma	1
Synovial sarcoma	2
Sclerosing epithelial fibrosarcoma	1
Spindle cell sarcoma with pleomorphic Liposarcoma	1
RT dose (Gy)	**N**
60	1
50	25
48	1
46.4	1
40	2
Unknown	8
% cells alive following RT	**N**
< 5	2
5 - 10	4
11 - 20	5
21 - 30	3
31 - 40	0
41 - 50	2
51 - 60	1
61 - 70	2
71 - 80	6
81 - 90	5
> 90	2
Unknown	6
Survival status following RT	**N**
Alive	20
Dead	10
Unknown	8

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Table 2

		up-regulation (N)	%	down-regulation (N)	%	*de novo (N)*	%	loss of expression (N)	%	no change (N)	%	ND (N)	%	N
A	**Immune cells**													
	CD45	19	51.4	3	8.1	0	0.0	0	0.0	15	40.5	0	0.0	37
	CD3	19	51.4	3	8.1	0	0.0	0	0.0	15	40.5	0	0.0	37
	CD4	21	56.8	3	8.1	0	0.0	0	0.0	13	35.1	0	0.0	37
	CD8	18	48.6	2	5.4	0	0.0	0	0.0	17	45.9	0	0.0	37
	MHC-I	20	54.1	0	0.0	0	0.0	0	0.0	17	45.9	0	0.0	37
	MHC-II	19	51.4	2	5.4	0	0.0	0	0.0	16	43.2	0	0.0	37
	β_2 microglobulin	20	54.1	2	5.4	0	0.0	0	0.0	15	40.5	0	0.0	37
	Perforin	21	56.8	4	10.8	2	5.4	0	0.0	9	24.3	1	2.7	37
	Granzyme B	13	35.1	4	10.8	0	0.0	0	0.0	9	24.3	11	29.7	37
	iNOS	6	16.2	8	21.6	2	5.4	0	0.0	6	16.2	15	40.5	37
	NKG2D	16	43.2	4	10.8	3	8.1	0	0.0	10	27.0	4	10.8	37
	Arginase 1	4	10.8	4	10.8	0	0.0	0	0.0	3	8.1	26	70.3	37
	BTLA	1	2.7	2	5.4	0	0.0	1	2.7	12	32.4	21	56.8	37
	CTLA-4	2	5.4	4	10.8	0	0.0	1	2.7	7	18.9	23	62.2	37
	FOXP3	5	13.5	13	35.1	0	0.0	1	2.7	10	27.0	8	21.6	37
	IDO	10	27.0	8	21.6	0	0.0	0	0.0	7	18.9	12	32.4	37
	PD-1	7	18.9	6	16.2	0	0.0	1	2.7	6	16.2	17	45.9	37
	PD-L1	7	18.9	6	16.2	0	0.0	0	0.0	16	43.2	8	21.6	37
B	**Cytokines**													
	IFN-Y	11	29.7	0	0.0	5	13.5	0	0.0	8	21.6	13	35.1	37
	TNF-α	11	29.7	8	21.6	0	0.0	0	0.0	16	43.2	2	5.4	37
	IL-12	5	13.5	1	2.7	2	5.4	0	0.0	5	13.5	24	64.9	37
	IL-10	4	10.8	11	29.7	0	0.0	1	2.7	15	40.5	6	16.2	37
	TGF-β	15	40.5	10	27.0	0	0.0	0	0.0	11	29.7	1	2.7	37
C	**Transcription factors**													
	STAT4	12	32.4	4	10.8	0	0.0	0	0.0	15	40.5	6	16.2	37
	STAT6	15	40.5	4	10.8	0	0.0	0	0.0	15	40.5	3	8.1	37
	STAT3	15	40.5	5	13.5	0	0.0	0	0.0	17	45.9	0	0.0	37
D	**CT-antigens**													
	CT7	11	29.7	1	2.7	5	0.0	0	0.0	3	8.1	17	45.9	37
	CT10	5	13.5	1	2.7	0	0.0	0	0.0	2	5.4	29	78.4	37
	LAGE-A1	16	43.2	0	0.0	1	0.0	0	0.0	2	5.4	18	48.6	37
	MAGE-A9	10	27.0	0	0.0	1	0.0	0	0.0	3	8.1	23	62.2	37
	NY-ESO-1	20	54.1	4	10.8	1	0.0	0	0.0	9	24.3	3	8.1	37
	PRAME	18	48.6	5	13.5	0	0.0	0	0.0	11	29.7	3	8.1	37
	SSX-2	0	0.0	0	0.0	0	0.0	0	0.0	2	5.4	35	94.6	37

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		up-regulation (N)	%	down-regulation (N)	%	*de novo* expression (N)	%	loss of expression (N)	%	no change (N)	%	ND (N)	%	N
A	**Immune cells**													
	CD3	26	70.3	1	2.7	0	0.0	0	0.0	10	27.0	0	0	37
	CD4	34	89.5	2	5.3	0	0.0	0	0.0	2	5.3	0	0	38
	CD8	26	68.4	1	2.6	0	0.0	0	0.0	11	28.9	0	0	38
B	**MHC-I**	30	78.9	0	0.0	0	0.0	0	0.0	8	21.1	0	0	38
C	**CT-antigens**													
	CT7	8	21.1	1	2.6	9	23.7	0	0.0	8	21.1	12	31.6	38
	NY-ESO-1	8	21.1	1	2.6	5	13.2	0	0.0	8	21.1	16	42.1	38